

UNITED STATES PATENT APPLICATION

of

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for

MULTIDRUG RESISTANCE PROTEINS IN DROSOPHILA AND ANOPHELES

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application is a continuation-in-part of prior Application No. 10/667,891, filed September 23, 2003. This application is based on and claims the benefit of U.S. Provisional Application S.N. 60/413,469, filed September 26, 2002 (Attorney Docket No. 03495.6079), and U.S. Application S.N. 10/667,891, filed September 23, 2003 (Attorney Docket No. 03495.0294), all of which are relied upon and incorporated by reference herein.

BACKGROUND OF THE INVENTION

[002] This invention relates to recombinant DNA technology. In particular, this invention concerns the cloning of nucleic acids encoding multiple drug resistance proteins of *Drosophila melanogaster* and *Anopheles gambiae*. More particularly, this invention provides isolated nucleic acid compounds encoding multiple drug resistance proteins of *Anopheles gambiae* and *Drosophila melanogaster*. Vectors and transformed host cells comprising the multiple drug resistance-encoding DNA of *Anopheles gambiae* and *Drosophila melanogaster* are also provided. The invention further provides assays, which utilize these transformed host cells.

[003] The multidrug-resistance associated protein, MRP1, a large (190 kDa) membrane glycoprotein, was identified in 1992 in a human small-cell lung cancer cell line where its overexpression conferred resistance to a large spectrum of drugs (Cole et al., 1992). MRP joined the P-glycoprotein MRP1, the original protein associated with broad resistance, discovered in 1976 (Juliano & Ling, 1976). The description of MRP1 was followed by the characterization of several other structurally related human

proteins: MRPs 2 to 8; followed by MRP9 (Bera et al., 2001; Büchler et al., 1996; Dean et al., 2001; Hopper et al., 2001). These proteins, like MRP1, are members of the ATP-binding cassette (ABC) superfamily, present from bacteria to man, and involved in the energy-dependent transmembrane transport of a variety of molecules, ranging from inorganic ions to large polypeptides. Like other ABC-transporters, MRP homologs are well conserved in evolution and have been described in several mammals (Büchler et al., 1996; Kool et al., 1997; van Aubel et al., 1998), in the nematode *Caenorhabditis elegans* (Broeks et al., 1996), in the protozoan parasite *Leishmania* (Essodaigui et al., 1999), in yeast (Szczypka et al., 1994), and in plants (Lu et al., 1997).

[004] ABC transporters are typically composed of two membrane spanning domains (MSDs), containing several transmembrane α -helices, and two cytosolic nucleotide binding domains (NBDs), responsible for the hydrolysis of ATP, thus providing the necessary energy for substrate transport. MRP1 is one of several members of the MRP subfamily characterized by a third MSD of unknown function at the N-terminus of the protein (Borst et al., 2000). The NBDs are highly conserved and share two sequence motifs, designated "Walker A" and "Walker B", with other nucleotide binding proteins. These sequences are separated by a stretch of about 120-170 amino acids, including a short (12-13 amino acid) peptide motif called the ABC transporter "signature" region. In contrast, the MSDs are highly divergent and are probably involved with the protein's substrate specificity.

[005] Notwithstanding these advances in the art, there continues to be a need in the art to identify MRPs in other species. For example, the identification of MRPs in insects could aid in the development of more effective insecticides.

SUMMARY OF THE INVENTION

[006] This invention describes the identification and characterization of a *Drosophila MRP* encoding a protein whose deduced amino acid sequence is closely related to that of human MRP1. (See Fig. 6.) Of particular interest is the identification of multiple copies of two exons (4 and 8), suggesting alternative splicing at the mRNA level. This hypothesis is supported by a RT-PCR strategy presented here.

[007] The *dMRP* of *Drosophila melanogaster* was the first description of a MRP homologue in an insect. Its deduced amino acid sequence is close (48% identity and 64.5% similarity) to that of the human MRP1 discovered in 1992 (Cole et al., 1992), and involved in the drug resistance of several tumors. These proteins belong to the ABCC1 subfamily of ATP-binding cassette (ABC) transporters, known to transfer a large variety of compounds across the cell membrane. Despite their close relatedness with other subgroups of ABC transporters, such as P-glycoproteins, CFTR, and sulfonylurea receptors, MRP proteins clearly form a separate cluster within this group, thus constituting an independent family (Borst et al., 1999).

[008] Extending these earlier findings, this invention also involves the discovery of the arrangement as a cluster of genes of the four copies of *MRP* homologues in

Anopheles gambiae, located on the chromosome 3R arm, and that all read on the same sense. This invention shows by RT-PCR that these potential genes are all transcribed in the adult mosquito, and seem ubiquitous in the different tissues. But their respective expressions are not always equivalent. The newly described genes are called *gMRP1a*, *gMRP1b*, *gMRP1c*, and *gMRP1d*. (See Fig. 6.) The three last have a close exon-intron structure (especially *gMRP1c* and *gMRP1d*), and share a strong homology and similarity, at the level of their amino acid sequences. In contrast, *gMRP1a* is much more different from the others regarding its sequence as well as structure, with only three introns in place of five in *gMRP1b*, and 6 in *gMRP1c* and *gMRP1d*.

[009] The invention provides, *inter alia*, isolated nucleic acid molecules that comprise nucleic acids encoding multiple drug resistance proteins from *Drosophila melanogaster* or *Anopheles gambiae*, herein referred to as *dMRP* or *gMRP*, respectively, vectors encoding *dMRP* or *gMRP*, and host cells transformed with vectors containing these nucleic acids.

[010] In another embodiment, the invention provides a method for determining the insecticidal MRP inhibition activity of a compound which comprises:

- a) placing a culture of insect cells, transformed with a vector capable of expressing *dMRP* or *gMRP*, in the presence of:
 - (i) an insecticidal agent to which said insect cell is resistant, but to which said insect cell is sensitive in its untransformed state;

- (ii) a compound suspected of possessing insecticidal MRP inhibition activity; and
- b) determining the insecticidal MRP inhibition activity of said compound by measuring the ability of the insecticidal agent to inhibit the growth of said insect cell.

[011] In still another embodiment, the present invention relates to strains of *Drosophila melanogaster* or *Anopheles gambiae* in which the *dMRP* or *gMRP* gene is disrupted or otherwise mutated such that the *dMRP* or *gMRP* protein is not produced in said strains.

[012] In yet another embodiment, the present invention relates to a method for identifying new insecticidal compounds.

[013] This invention further provides isolated polynucleotides that comprise an isolated DNA sequence encoding SEQ ID NOS: 1, 2, 3, 4, or 5, or a vector containing the isolated polynucleotide.

[014] The isolated polynucleotide of the invention, or a portion thereof, can be labeled with a detectable moiety.

[015] A host cell containing the vector of the invention is also provided.

[016] A method for constructing a transformed host cell capable of expressing SEQ ID NOS: 1, 2, 3 4, or 5 comprises transforming a host cell with a recombinant DNA vector that comprises an isolated DNA sequence of the invention.

[017] A method for expressing SEQ ID NOS: 1, 2, 3, 4 or 5 in a transformed host cell comprises culturing the transformed host cell of the invention under conditions suitable for gene expression.

BRIEF DESCRIPTION OF THE DRAWINGS

[018] This invention will be described in greater detail in the drawings in which:

[019] **FIG. 1.** Comparison of deduced *D. melanogaster* cDNA SD07655 and human MRP1 amino acid sequences. The two amino acid sequences were aligned using ClustalW. Identical residues are marked with shading. The transmembrane regions are noted by a fine underline and the ATP-binding domains are noted by a bold underline. The amino acids derived from exons 4 and 8 of the dMRP gene are presented in bold characters. The small vertical lines above and below the amino acids denote the exon junctions with the type of splice junction marked by a number noting the class: 0, 1 or 2. The dMRP amino acid sequence differs from that of sequence AY069827 at the following positions : L/V pos. 124, M/L pos. 318 and I/T pos. 448.

[020] **FIG. 2.** Genomic organization of *dMRP* and alignment of the splice junctions with dMRP cDNA SD07655. The intron-exon organization of *dMRP* is illustrated at the bottom with the exons indicated by the vertical bars and introns by the thin horizontal lines. The regions of the *dMRP* mRNA encoded by each exon are shown at the top. Superimposed on the mRNA is a schematic of the protein with membrane spanning domains (MSDs), and nucleotide binding domains (NBDs). Within each MSD,

the transmembrane helices are shown as black bars. Walker A and Walker B are designated as A and B, respectively, and C indicates the ABC family signature.

[021] **FIG. 3.** Expression of exons 4 and 8 variants in cellular RNA. Total cDNA was prepared as described in Materials and Methods. A first nonspecific PCR amplification of the exon 8 (A) or 4 (B) was generated using primers in neighboring exons. The presence of cDNA from a specific exon was tested using one nonspecific primer and a second primer that represented a specific sequence in the exon being tested. All of the primer sequences and the predicted product sizes are listed in Table 2. (A) The exon 8 region of the cDNA was amplified using primers DR17/DR18. The specific amplifications used primer rev8 and the specific primer 8a - 8g. (B) The MRP exon 4 region of the cDNA was amplified with primers Dr2for and Dr6rev. The exon 4 specific reactions used primer sets Dr2for/Dr4arev (lanes A, B, C) or Dr2for/Dr4brev (lanes D, E, F). The DNA targets were the product of reaction Dr2for/Dr6rev (lanes A and D), cDNA SD07655 (lanes B and E), *Anopheles gambiae* genomic DNA (lanes C and F). The PCR products were separated on a 1% agarose gel using the molecular weight marker (MW) SmartLadder SF (Eurogentec; Seraing, Belgium).

[022] **FIG. 4.** Amino acid alignment of dMRP variable exon 4 (A) and 8 (B) encoded peptides with the cognate peptides from other organisms. The variant dMRP peptide sequence and the equivalent sequences from *Drosophila* sulfonylurea receptor (Dsur, NG_000795) and three human MRPs (MRP1, NM_004996; MRP2, NP_005836; and MRP3, Y17151) were aligned using ClustalW. Pfam refers to pfam00664, a

consensus sequence for ABC transporter Membrane Spanning Domains. Gaps were introduced to maximize sequence identity and are shown by a horizontal dash. Residues that are identical in at least half of the sequences have their background shaded and those present in more than half of the sequences are listed in the consensus (Cons). (C) Dendrogram constructed with the data of part (B) of the Figure (see *infra* for details).

[023] **Fig. 5.** Schematic representation of the four *gMRP* genes showing overall organization of exons and introns. Exons appear in black and are numbered under the sequence, while introns appear in white and are numbered in italic above the sequence.

[024] **Fig. 6.** Comparison of deduced *A. gambiae* gMRP1a-d, *Drosophila melanogaster* dMRP, and human MRP1 amino acid sequences. The alignment was produced using ClustalW. Identical residues in at least half of the sequences are marked with shading. The different topological regions are indicated in bold and italic above the sequences, and are delimited by vertical bars. *MSD1-3*, Membrane Spanning Domains 1 to 3; *L₀*, cytoplasmic loop; *NBD1-2*, Nucleotide Binding Domain, *Linker*, region linking the two halves of the protein. Walker A and Walker B are indicated as *A* and *B*, and their sequences are marked in bold, as well as the signature (*C*) of ABC transporters. The vertical lines in bold inside the amino acid sequences denote the exon junctions. Where several genes shared the same site, this one was emphasized by a delimitating box.

DETAILED DESCRIPTION OF THE INVENTION

[025] At the beginning of 2001, an international consortium organized the *A. gambiae* genome project, which now comes in line with the sequencing of the *Plasmodium* and the human genomes. Before the release of the whole sequence on (a web site) preliminary work on a BAC clone library was generated by Frank H. Collins, Univ. of Notre Dame, USA. The BAC library inserts ends were sequenced by the Genoscope (Evry, France) and representing the anopheline genome, allowed the finding of four copies of homologues of the *Drosophila* and human MRPs, which were further characterized. The study of the *Drosophila* gene had put in light a new splicing process in comparison to previously examined MRPs in several animals. Two exons are present as multiple copies, which potentially allows the insect to express as many as 14 different variants of the protein. This peculiar process was not retrieved in *A. gambiae*.

[026] The present invention provides isolated nucleic acid molecules that comprise a nucleic acid sequence encoding dMRP or gMRP. The amino acid sequence of the protein encoded by dMRP or gMRP is provided in the Sequence Listing as SEQ ID NO: 3 and SEQ ID NOs: 2-5, respectively. As shown in Fig. 6, gMRP1a, gMRP1b, gMRP1c, and gMRP1d are identified as SEQ ID NOS: 2-5, respectively. SEQ ID NO: 1 identifies dMRP, and MRP1 is identified as SEQ ID NO: 6.

[027] Those skilled in the art will recognize that the degenerate nature of the genetic code enables one to construct many different nucleic acid sequences that encode the amino acid sequences of SEQ ID NO: 1-5. Consequently, the constructions

described below and in the accompanying examples for the preferred nucleic acid molecules, vectors, and transformants of the invention are illustrative and are not intended to limit the scope of the invention. All nucleotide and amino acid abbreviations used in this disclosure are those accepted by the United States Patent and Trademark Office as set forth in 37 C.F.R. §1.822(b) (1994).

[028] The term "vector" refers to any autonomously replicating or integrating agent, including but not limited to plasmids, cosmids, and viruses (including phage), comprising a nucleic acid molecule to which one or more additional nucleic acid molecules can be added. Included in the definition of "vector" is the term "expression vector". Vectors are used either to amplify and/or to express deoxyribonucleic acid (DNA), either genomic or cDNA, or RNA (ribonucleic acid), which encodes dMRP or gMRP, or to amplify DNA or RNA that hybridizes with DNA or RNA encoding dMRP or gMRP.

[029] The term "expression vector" refers to vectors, which comprise a transcriptional promoter (hereinafter "promoter"), and other regulatory sequences positioned to drive expression of a DNA segment that encodes dMRP or gMRP. Expression vectors of the present invention are replicable DNA constructs in which a DNA sequence encoding dMRP or gMRP is operably linked to suitable control sequences capable of effecting the expression of dMRP or gMRP in a suitable host. Such control sequences include a promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and

sequences that control termination of transcription and translation. DNA regions are operably linked when they are functionally related to each other. For example, a promoter is operably linked to a DNA coding sequence if it controls the transcription of the sequence, or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

[030] The term "MRP inhibition activity" refers to the ability of a compound to inhibit the MRP activity of a host cell, thereby increasing the insecticidal activity of an insecticidal compound against said host cell.

[031] In the present invention, dMRP or gMRP may be synthesized by host cells transformed with vectors that provide for the expression of DNA encoding dMRP or gMRP. The DNA encoding dMRP or gMRP can be the natural sequence or a synthetic sequence or a combination of both ("semi-synthetic sequence"). The *in vitro* or *in vivo* transcription and translation of these sequences results in the production of dMRP or gMRP. Synthetic and semi-synthetic sequences encoding dMRP or gMRP can be constructed by techniques well known in the art. See Brown et al. (1979) Methods in Enzymology, Academic Press, N.Y., 68:109-151. dMRP or gMRP -encoding DNA, or portions thereof, can be generated using a conventional DNA synthesizing apparatus, such as the Applied Biosystems Model 380A, 380B, 394 or 3948 DNA synthesizers (commercially available from Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, Calif. 94404).

[032] Owing to the natural degeneracy of the genetic code, the skilled artisan will recognize that a sizable yet definite number of nucleic acid sequences can be constructed that encode dMRP or gMRP. All such nucleic acid sequences are provided by the present invention. These sequences can be prepared by a variety of methods and, therefore, the invention is not limited to any particular preparation means. The nucleic acid sequences of the invention can be produced by a number of procedures, including DNA synthesis, cDNA cloning, genomic cloning, polymerase chain reaction (PCR) technology, or a combination of these approaches. These and other techniques are described by Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989), or *Current Protocols in Molecular Biology* (F. M. Ausubel et al., 1989 and supplements). The contents of both of these references are incorporated herein by reference.

[033] In another aspect, this invention provides the genomic DNA encoding dMRP or gMRP, which may be obtained by synthesizing nucleic acids encoding the desired portion of SEQ ID NOS: 1-5 or by following the procedure described in the Examples.

[034] To effect the translation of dMRP or gMRP-encoding mRNA, one inserts the natural, synthetic, or semi-synthetic dMRP or gMRP-encoding DNA sequence into any of a large number of appropriate expression vectors through the use of appropriate restriction endonucleases and DNA ligases. Synthetic and semi-synthetic dMRP or gMRP-encoding DNA sequences can be designed, and natural dMRP or gMRP-

encoding nucleic acid can be modified to possess restriction endonuclease cleavage sites to facilitate isolation from and integration into these vectors. Particular restriction endonucleases employed will be dictated by the restriction endonuclease cleavage pattern of the expression vector utilized. Restriction enzyme sites are chosen so as to properly orient the dMRP or gMRP-encoding DNA with the control sequences to achieve proper in-frame transcription and translation of the dMRP or gMRP molecule. The dMRP or gMRP-encoding DNA must be positioned so as to be in proper reading frame with the promoter and ribosome binding site of the expression vector, both of which are functional in the host cell in which dMRP or gMRP is to be expressed.

[035] Expression of dMRP or gMRP can be accomplished in cells, such as *Saccharomyces cerevisiae*. Suitable promoter sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (found on plasmid pAP12BD (ATCC 53231) and described in U.S. Pat. No. 4,935,350, Jun. 19, 1990) or other glycolytic enzymes, such as enolase (found on plasmid pAC1 (ATCC 39532)), glyceraldehyde-3-phosphate dehydrogenase (derived from plasmid pHcGAPC1 (ATCC 57090, 57091)), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Inducible yeast promoters have the additional advantage of transcription controlled by growth conditions. Such promoters include the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism,

metallothionein (contained on plasmid vector pCL28XhoLHBPV (ATCC 39475), U.S. Pat. No. 4,840,896), glyceraldehyde 3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization (GAL1 found on plasmid pRY121 (ATCC 37658) and on plasmid pPST5, described below). Suitable vectors and promoters for use in yeast expression are further described by R. Hitzeman et al., in European Patent Publication No. 73,657A. Yeast enhancers, such as the UAS Gal enhancer from *Saccharomyces cerevisiae* (found in conjunction with the CYC1 promoter on plasmid YEpsec--hl1beta, ATCC 67024), also are advantageously used with yeast promoters.

[036] A variety of expression vectors useful in the present invention are well known in the art. For expression in *Saccharomyces*, the plasmid YRp7, for example, (ATCC-40053, Stinchcomb et al., 1979, *Nature* 282:39; Kingsman et al., 1979, *Gene* 7:141; Tschemper et al., 1980, *Gene* 10:157) is commonly used. This plasmid contains the trp gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC 44076 or PEP4-1 (Jones, 1977, *Genetics* 85:12).

[037] Expression vectors useful in the expression of dMRP or gMRP can be constructed by a number of methods. For example, the cDNA sequence encoding dMRP or gMRP can be synthesized using DNA synthesis techniques, such as those described above. Such synthetic DNA can be synthesized to contain cohesive ends that allow facile cloning into an appropriately digested expression vector. For example,

the cDNA encoding dMRP or gMRP can be synthesized to contain NotI cohesive ends. Such a synthetic DNA fragment can be ligated into a NotI-digested expression vector such as pYES-2 (Invitrogen Corp., San Diego Calif. 92121).

[038] The techniques involved in the transformation of yeast cells, such as *Saccharomyces cerevisiae* cells, are well known in the art and may be found in such general references as Ausubel et al., Current Protocols in Molecular Biology (1989), John Wiley & Sons, New York, N.Y. and supplements. The precise conditions under which the transformed yeast cells are cultured is dependent upon the nature of the yeast host cell line and the vectors employed.

[039] Nucleic acid, either RNA or DNA, which encodes dMRP or gMRP, or a portion thereof, is also useful in producing nucleic acid molecules useful in diagnostic assays for the detection of dMRP or gMRP mRNA, dMRP or gMRP cDNA, or dMRP or gMRP genomic DNA. Further, nucleic acid, either RNA or DNA, which does not encode dMRP or gMRP, but which nonetheless is capable of hybridizing with dMRP or gMRP-encoding DNA or RNA is also useful in such diagnostic assays. These nucleic acid molecules can be covalently labeled by known methods with a detectable moiety, such as a fluorescent group, a radioactive atom, or a chemiluminescent group. The labeled nucleic acid is then used in conventional hybridization assays, such as Southern or Northern hybridization assays, or polymerase chain reaction assays (PCR), to identify hybridizing DNA, cDNA, or RNA molecules. PCR assays can also be performed using unlabeled nucleic acid molecules. Such assays can be employed to identify dMRP or

gMRP vectors and transformants and in *in vitro* diagnosis to detect *dMRP* or *gMRP*-like mRNA, cDNA, or genomic DNA from other organisms.

[040] Compounds with demonstrated insecticidal activity can be potentiated by an MRP inhibitor such that the insecticidal activity of these compounds is extended to previously resistant species. To identify compounds useful in such combination the present invention provides an assay method for identifying compounds with *Anopheles gambiae* or *Drosophila melanogaster* MRP inhibition activity. Host cells that express dMRP or gMRP provide an excellent means for the identification of compounds useful as inhibitors of *Anopheles gambiae* or *Drosophila melanogaster* MRP activity.

Generally, the assay utilizes a culture of a cell transformed with a vector that provides expression of dMRP or gMRP. The expression of dMRP or gMRP by the host cell enables the host cell to grow in the presence of an insecticidal compound to which the cell is sensitive to in the untransformed state. Thus, the transformed yeast cell culture is grown in the presence of i) an insecticidal agent to which the untransformed cell is sensitive, but to which the transformed host cell is resistant, and ii) a compound that is suspected of being an MRP inhibitor. The effect of the suspected MRP inhibitor is measured by testing for the ability of the insecticidal compound to inhibit the growth of the transformed cell. Such inhibition will occur if the suspected *Anopheles gambiae* or *Drosophila melanogaster* MRP inhibitor blocks the ability of dMRP or gMRP to prevent the insecticidal compound from acting on the cell. The *Drosophila Schneider* cell can be used for example. The *Drosophila Schneider* cell line, for example, can be used.

[041] Oligonucleotides are provided by this invention, which are specifically hybridizable with nucleic acids encoding the dMRP and gMRP. Also provided are methods of using the oligonucleotides of the invention in methods of modulating the expression of MRP genes, inhibition of which leads to inhibition of the synthesis of dMRP and gMRP, and thereby inhibits cellular multidrug resistance. Such inhibition is desirable for preventing or modulating the development of multidrug resistance in an insect. Modified derivatives of the oligonucleotides of the invention, such as chimeras and conjugates (e.g., of an oligonucleotide and a lipophilic moiety, such as cholesterol), are also contemplated by the invention. The biological activity and cellular uptake of oligonucleotides is enhanced by such modifications.

[042] In accordance with the present invention oligonucleotides are provided, which specifically hybridize with nucleic acids encoding an dMRP or gMRP. Certain oligonucleotides of the invention are designed to bind either directly to mRNA transcribed from, or to a selected DNA portion of, *dMRP* or *gMRP*, thereby modulating the amount of protein translated from *dMRP* or *gMRP* mRNA or the amount of mRNA transcribed from *dMRP* or *gMRP*, respectively.

[043] Oligonucleotides can comprise nucleotide sequences sufficient in identity and number to effect specific hybridization with a particular nucleic acid. Such oligonucleotides are commonly described as "antisense." Antisense oligonucleotides can be used as research tools and diagnostic aids. MRP genes encoding dMRP or gMRP are particularly useful for this approach. Inhibition of the expression of such

MRP genes leads to inhibition of the synthesis of dMRP or gMRP and thereby inhibits cellular multidrug resistance. Such inhibition is desirable for preventing or modulating the development of multidrug resistance in an insect. The specific hybridization exhibited by the oligonucleotides of the present invention can be used for assays, purifications, cellular product preparations and in other methodologies that will be appreciated by persons of ordinary skill in the art.

[044] Methods of modulating the expression of dMRP and gMRP comprising contacting insects with oligonucleotides specifically hybridizable with an MRP gene are herein provided. These methods are useful as a consequence of the association between MRP expression and the multidrug resistance of cells. These methods are also useful as tools, for example, in the detection and determination of the role of dMRP and gMRP expression in various cell functions and physiological processes and conditions, and for the diagnosis of conditions.

[045] The present invention employs oligonucleotides for use in antisense inhibition of the function of RNA and DNA encoding proteins. The present invention also employs oligonucleotides that are designed to be specifically hybridizable to DNA or messenger RNA (mRNA) encoding dMRP and gMRP and ultimately modulating the amount of such proteins transcribed from their respective MRP genes. Such hybridization with mRNA interferes with the normal role of mRNA and causes a modulation of its function in cells. The functions of mRNA to be interfered with include all vital functions, such as translocation of the RNA to the site for protein translation,

actual translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with mRNA function is modulation of the expression of such dMRP and gMRP. In the context of this invention, “modulation” means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression.

[046] Oligonucleotides comprise nucleotide sequences sufficient in identity and number to effect specific hybridization with a particular nucleic acid. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are useful for elucidating the function of particular genes, for example, to distinguish between the functions of various members of a biological pathway. This specific inhibitory effect has, therefore, been harnessed for research use.

[047] The oligonucleotides in accordance with this invention preferably comprise from about 8 to about 30 nucleotides. It is more preferred that such oligonucleotides comprise from about 15 to 25 nucleotides.

[048] The present invention employs oligonucleotides targeted to nucleic acids encoding dMRP and gMRP, and oligonucleotides targeted to nucleic acids encoding such proteins. Kits for detecting the presence or absence of MRP expression can also be prepared. Such kits include an oligonucleotide targeted to an MRP gene encoding a dMRP and gMRP. Such kit and assay formats are known in the art.

[049] “Complementary,” as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other.

[050] Thus, “specifically hybridizable” and “complementary” are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that an oligonucleotide need not be 100% complementary to its target DNA sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed.

DROSOPHILA MELOGANSTER

Materials and methods

Genomic DNA extraction

[051] Adult *D. melanogaster* (1.5 g) were homogenized in liquid nitrogen. After addition of 7 ml of lysis buffer (100 mM Tris pH 8, 50 mM NaCl, 50 mM EDTA, 1% SDS, 0.15 mM spermine, and 0.5 mM spermidine) plus proteinase K (100 µg/ml), the homogenate was mixed slowly at 37°C for 2 H. Following extraction with phenol (1X), with chloroform (1X) and dialysis overnight at 4°C against TEN buffer (500 mM Tris pH 8, 10 mM EDTA, 10 mM NaCl), the DNA was treated with RNase (DNase free, 100 ng/ml) at 37°C for 1H. It was then extracted once with phenol/chloroform (1:1), with chloroform, and dialyzed overnight at 4°C against TE buffer (10 mM Tris pH 8, 1 mM EDTA). The DNA was then precipitated with 1/10 volume of 3 M sodium acetate and two volumes absolute ethanol, collected, redissolved, and its final concentration was estimated on an agarose gel. DNA was prepared from larvae and pupae by the same method.

Southern blots

[052] DNA (10 µg) was digested with either *Bam*HI or *Hind*III and the fragments were separated by electrophoresis on a 0.8% agarose gel. Following transfer to Hybond-N nylon membrane and fixation, hybridization was carried out at 65°C (in 1% BSA, 0.25 M NaH₂PO₄ pH 7.2, 1 mM EDTA, 150 µg/ml salmon sperm DNA) with a

PCR-derived *dMPP* probe covering 378 bases (forward primer: GATCCGTTTATTTCTTGCCGC; reverse primer: TCCAGGGCAGTGATTACCAGT). After hybridization, the blot was washed (in 40 mM NaH₂PO₄ pH 7.2, 1% SDS, and 1 mM EDTA) 1X at RT and 2X at 65 C°.

Reverse transcriptase-PCR (RT-PCR)

Extraction of total RNA and cDNA synthesis

Total RNA was isolated from 0-4 H eggs, 12-24 H eggs, pupae, and adult [053] *D. melanogaster* using Tri Reagent (Sigma) according to the manufacturer's instructions. The cDNA was synthesized in a 20 µl reaction mix containing 10 µg of total RNA, 1X AMV reverse transcriptase buffer (Promega), 10 U of AMV reverse transcriptase (Promega), 40 U of RNasin (Promega), 4 µg of random hexanucleotide primers (Genset), and 1 µM of each dNTP (Pharmacia). The reaction was incubated at 37°C for 1 H, followed by 95°C for 5 min.

Amplification of exon 4

[054] The first PCR used primers Dr2for/Dr6rev (Table 2) and 1 µl of cDNA as template. The cycling conditions were 94°C (3 min) for 1 cycle; 94°C (45 sec), 60°C (30 sec) and 72°C (90 sec) for 35 cycles; 72°C (10 min) for 1 cycle. This amplified product was used as template (0.5 µl) for a second PCR with the same cycling conditions with primer set specific for exon 4a or 4b (Table 2).

Amplification of exon 8

[055] Essentially the same conditions as those described for exon 4 were used for exon 8 containing cDNA, except that the first primer set was DR17/DR18 (Table 2) and the annealing temperature was 55°C. After electrophoresis on a 1% agarose gel, the amplified DNA was excised, purified using a Jetsorb kit (Genomed) and used as template for a second PCR with the same cycling conditions but with the exon 8 primer sets designated in Table 2.

DNA sequencing

[056] PCR-amplified DNA was purified using the Qiaquick PCR purification kit (Qiagen) and sequenced using an ABIprism 310 automated DNA sequencer. The cDNA clone SD07655 was sequenced directly from the plasmid after amplification in *E. coli* DH5α bacteria and extraction with a Qiafilter Plasmid Midi kit (Qiagen). Kits were used according to the manufacturer's instructions.

Sequence analysis

[057] The BLAST searches were done using the Washington University (Gish, W., 1996-2002 <<<http://blast.wustl.edu>>>) and the NCBI (Altschul et al., 1997) versions of BLAST. ClustalW (Higgins & Sharp, 1988) or PileUp from the Wisconsin Package programs licensed from the Genetics Computer Group (GCG) was used for sequence alignments. The TMAP and Needle programs, used for transmembrane domain search

and pairwise sequence comparisons, are part of the EMBOSS software package from EMBL. The software, except for the GCG package, were accessed through the Institut Pasteur web site at <<http://bioweb.pasteur.fr>>. Many common DNA and protein sequence manipulations were done with DNA Strider, a Macintosh DNA program (Marck, 1988).

Characterization of the Drosophila MRP

[058] Before the release of the annotated *Drosophila* genome, the amino acid sequence of human MRP1 was compared to the available genomic sequences using the TBLASTN program through the Berkeley *Drosophila* Genome Project (BDGP) web site (<http://www.fruitfly.org>). The BAC clone AC005819 was identified as containing a sequence that could code for a protein having high similarity with the human MRP1. The concerned region of the clone was then scanned against the available *Drosophila* Expressed Sequence Tags (ESTs), and several cDNA sequences were detected. Clone SDO7655 was selected, obtained from Research Genetics (Huntsville, AL, USA) and sequenced. During the preparation of this report, BDGP has independently released the SD07655 cDNA sequence, accession number AY069827, for gene FBgn0032456. The gene encoding SD07655 has been given the name CG6214 by the BDGP.

[059] When the deduced protein sequence of SD07655 was compared to those of all predicted *Drosophila* proteins, it naturally was most similar to the predicted product of gene CG6214, the gene from which SD07655 was transcribed. The next best

predicted protein, the CG14709 product, has a blastp bit score of only 747 compared to the 2,937 score for that of CG6214. Likewise, if the human MRP1 amino acid sequence is used as the query, the bit score with our deduced SD07655 amino acid sequence (1,484) is twice as high as with the next best sequence derived from gene CG14709 (770). These results argue that CG6214 is a unique gene and is the *Drosophila* homolog the most related to human *MRP1*. This proposal was confirmed by a Southern blot of *Bam*HI- or *Hind*III-digested genomic adult fly DNA probed with a SD07655 PCR product (data not shown).

[060] The deduced 1548 amino acid sequence generated from the SD07655 nucleotide sequence is shown in Fig. 1. The actual initiation methionine is not known, but the selected one was chosen based on a positional comparison with animal members of the MRP family, and because it was preceded by stop codons in all three ORFs, as well as having a consensus A at position – 3 and a G at position + 4, as discussed by Kozak (1996). The deduced amino acid sequence of this *Drosophila* cDNA produced an alignment with 48% identity and 64.5% similarity with the human MRP1 protein, and more than 44% identity and 60% similarity with several other MRPs (Table 1).

TABLE 1. Identity and similarity between dMRP and some other MRPs^a

	Percent identity/similarity				
	MRP1	MRP2	MRP3	CeMRP1	Dsur
dMRP	48 / 64.5	44.7 / 63.4	47.7 / 63.6	44.1 / 61.2	23.7 / 38.0
MRP1		48.7 / 67.0	57.1 / 74.1	46.7 / 64.6	24.5 / 38.3
MRP2			47.2 / 66.3	42.3 / 61.6	23.4 / 37.8
MRP3				44.3 / 63.1	24.1 / 38.0
CeMRP1					24.4 / 38.1

(a) Percent identity and similarity between the sequence pairs was calculated using the Needle program. The amino acid sequences are as follows : dMRP, *D.melanogaster*; MRP1, human (NM_004996); MRP2, human (NP_005836); MRP3, human (Y17151); CeMRP1, *C.elegans* (AB023045); Dsur, *D.melanogaster* sulfonylurea receptor (NG_000795).

[061] Topology predictions and comparison with other characterized MRPs point to six main regions on the mRNA, corresponding to the following parts of the protein : three MSDs (MSD 1-3), two NBDs (containing motifs A, C, B), and a linker region connecting NBD1 to MSD3 (Fig. 1 and 3). The NH₂-proximal MSD1 could span the membrane three times, while MSDs 2 and 3 may each contain four transmembrane helices, and precede NBDs 1 and 2, respectively. As seen in the alignment with the human MRP1 (Fig. 1), the similarity is especially prominent in the NBDs (706-855 and 1368-1527) and to a lesser extent in MSDs 2 and 3 (355-619 and 1020-1281). By contrast, MSD1 (110-254) and the linker region, between NBD1 and MSD3, are poorly conserved, a usual feature in MRPs. The Walker A and B boxes are easily identified in the NBDs, as well as the ABC signature. As with other MRPs, the first NBD of dMRP is 13 amino acids smaller than that of P-glycoproteins (Cole et al., 1992; Deeley & Cole, 1997).

[062] A simple comparison of the SD07655 cDNA and the genomic sequences defined a gene that spans slightly more than 22 kbp and contains 12 exons. Closer examination showed that exons 4 and 8 are represented by two and seven similar potential exons, respectively. Fig. 2 graphically depicts the predicted relationship between the cDNA and genomic sequences. This arrangement predicts a 12 exon cDNA including one copy each of the exon 4 and 8 variants as is seen with cDNA SD07655. The only important differences between this model and the FlyBase annotation are the inclusion of two copies of exon 4 in their predicted sequence, plus the predicted use of exon 8a rather than exon 8d, present in cDNA SDO7655. Their use of exon 8a is justified by the occurrence of EST LD28149, which begins in that exon sequence.

Table 2. PCR Primers and Their Expected Product Sizes

SEQ ID NO:	Primer name	Primer sequence	Size of PCR product expected with Dr2for (bp)	Size of PCR product expected with DR17 (bp)	Size of PCR product expected with rev8 (bp)
29	Dr2for	AGTGATTGCCAGTCGCATCA			
30	Dr6rev	GCCGTTCTCAATGCTCATTG	493		
31	Dr4arev	CTCGGCTATGTCAACACTCA	382		
32	Dr4brev	TTGCACCCAGGTTGGTGATCA	382		
33	DR17	AACGATCAAAATGTCGCC			
34	DR18	CACGAATAGTCGATGCTCC		500	
35	rev8	GGGAATTCGCGTGACAGACTAAT			
36	8a	GGGAATTCGCGACGAACTTCTCTC			269
37	8b	GGGAATTCCTTACCTCGTACTCTTTTG			269
38	8c	GGGAATTCCTTGTACAGGGTATCTATC			269
39	8d	GGGAATTCCTATCCAAATATTTATCGGGG			269
40	8e	GGGAATTCGTTTCACGTCATTCCTTTTC			272
41	8f	GGGAATTCGTCCTTTTGCAATTACGGCGC			269
42	8g	GGGAATTCGTGTGCTAGCCTACTTTTGC			269

[063] To assess which of the predicted exon 4 and 8 variants are used in mRNA, their expression was measured by a nested RT-PCR technique (Materials and methods, section 2.3). Fig. 3 shows that all seven alternative versions of exon 8, and the two variants of exon 4 were detected at all life stages tested, suggesting that use of the different exons is not developmentally regulated. Importantly, the sizes of the PCR products are those expected if only one copy of each exon is included in the cDNA. The accuracy of the amplification was verified by purification and sequencing of the products. In the case of exon 4, each product had a band slightly larger than the main band after the second PCR, even when the first PCR product was purified. Although we can not explain this phenomenon, sequencing the total product in both directions unequivocally resulted in the predicted sequence, either 4a or 4b.

Analysis of intron-exon boundaries

[064] To determine the intron-exon borders, the cDNA and genomic sequences were compared. The sizes of the introns and exons and their locations are shown in Table 3 along with the intron-exon junction sequences. The size of the exons varies from 85 bp for exon 5 to 1,512 bp for exon 2, with an average around 340 bp. The mean size for the introns is 867 bp, the largest being introns 13 and 18 which are 4,965 bp and 4,791 bp, respectively. The shortest intron is number 17 with 59 bp. Compared to the human *MRP1* organization, the *Drosophila* exons are larger and the introns smaller (Grant et al., 1997). The intron-exon boundaries (Table 3) are in accordance with those of other eukaryotic genes, especially those of human *MRP1*. The acceptor is

characterized by a (t/c)ag sequence in 84% of the cases, exons 4a and 8b being the only exceptions with an aag sequence. The splice donor sequences are G/gt(g/a)a(g/t), with a G in 83%, gt(g/a) in 100%, a in 78%, and (g/t) in 83% of the cases. Of the 18 introns, 13 (72%) are class 0, 2 (11%) are class 1, and 3 (17%) are class 2. In comparison, *MRP1* introns are class 0 in 63% of the cases, 10% are class 1, and 27% are class 2. This clearly demonstrates a mutual bias toward class 0 introns in these genes, though *MRP1* uses more class 2 introns than *dMRP*. The importance of this difference is difficult to interpret, nevertheless, it is interesting to note that four splice junctions and their class are conserved between the two genes (Fig. 1). The variable exon 4, located just before the NBD1 region, has been conserved between the two genes based on both their splice junctions, conserved amino acid sequence and exon length. Also, the initial *dMRP* exon 8 splice junction has been conserved in human *MRP1* despite the relative divergence in this region.

TABLE 3. Intron-exon organization of the *Drosophila* dMRP gene

Exon					Intron		
n°	Size (bp)	3' acceptor ^a	exon location ^b	5' donor	n°	Phase	Size (bp)
1	181		-127•54	TTCTGG / gtgagt	1	0	74
2	1512	gaacag / AACGCA	129•1640	ATTAAG / gtgagt	2	0	135
3	138	acatag / GTGCTC	1776•1913	TTCCTG / gtaaga	3	0	128
4a	147	acaaag / GTTTCC	2042•2188	GCCGAG / gtacag	4	0	146
4b	147	ttttag / GTTTCA	2335•2481	GTGCAA / gtaagt	5	0	800
5	85	gaatag / ACGCAA	3282•3366	CTAAAC / gtaaga	6	1	62
6	820	atacag / CCCATC	3429•4248	TTCAT / gtaagt	7	2	67
7	371	ttttag / CTCCGT	4316•4686	GCCAAG / gtaagt	8	1	904
8a	221	ttctag / TCGCGA	5591•5811	TATATG / gtaatt	9	0	336
8b	221	tcgaag / TTGTTA	6148•6368	TTTGCG / gtaatt	10	0	385
8c	221	ttccag / TTACCT	6754•6974	TTTGCG / gtaaat	11	0	525
8d	221	atgcag / TGCTAT	7500•7720	TTCGGG / gtaaaag	12	0	691
8e	224	tccag / GTGTGC	8412•8635	TTTATG / gtattt	13	0	4965
8f	221	agctag / GTCTTT	13605•13825	TTTCAG / gtaatc	14	0	1141
8g	221	tcgcag / GTTTCA	14967•15187	TTCGAG / gtaatt	15	0	340
9	218	ggttag / GTTCTG	15528•15745	AGATCG / gtatgt	16	2	64
10	507	cttcag / CTTTAT	15810•16316	GTTCAG / gtaagc	17	2	59
11	382	atttag / AATAAT	16376•16757	ATTCAG / gtgggt	18	0	4791
12	393	ctatag / AAAACC	21549•21941				

Comparison of the variable exon encoded sequences

[065] Six of seven exon 8 variants encode a 74 amino acid peptide while exon 8e encodes one additional amino acid. Likewise, the two exon 4 variants are the same length and use the same splice junction types. Alignment of the amino acid sequences encoded by the different exon 4 variants with the corresponding sequences from human MRPs 1-3 and the *D. melanogaster* sulphonylurea receptor (SUR) are shown in Fig. 4A. The two dMRP peptides are nearly identical in their N-terminal third, but the 4a derived peptide varies extensively from both the 4b and the human sequences in the otherwise highly conserved middle third of the peptide. This pattern suggests that

conserved amino acids 18-37 may be important in normal MRP function and that the variation in this region of 4a gives the protein new properties.

[066] When exon 8 variation is examined (Fig. 4B), there is a highly conserved region from amino acids 30-61 with the most divergence in variant 8e. The “FF” motif at positions 36-37 in the alignment is highly conserved in all the human sequences as well as in SUR and Pfam, the Protein family database consensus sequence (Bateman et al., 2002), but is only maintained in variants 8e and 8f. On the other hand, the exon 8 variants favor a Leu or Met in place of the second Phe. The extremities of the region are relatively divergent amongst all the proteins, suggesting that sequence conservation is less important in these regions.

[067] To clarify the level of sequence similarity between the exon 8 variants and the corresponding region of other related proteins, a dendrogram was constructed using the UPGMA method on a matrix calculated with the PUZZLE program (Strimmer & von Haeseler, 1996). The dendrogram (Fig. 4C) shows the exon 8 heterogeneity: variants 8a, 8b, 8c, and 8d group together while 8g clusters with MRP2, and 8f and 8e are separated from the others. The Figure shows that while overall the *Drosophila* protein is very similar to human MRP1, in the exon 8 encoded region there is a great deal of diversity. This diversity could be related to the different substrates encountered by these transporters in their respective environments.

Conclusions

[068] While alternative splicing has previously been described in the human *MRPs*, those cases differ from the type of splicing described in this article since previous reports detail the deletion of a terminal exon from the major product (Fromm et al., 1999; Grant et al., 1997) or the use of alternate transcriptional start sites (Bera et al., 2001; Suzuki et al., 2000). In the case of dMRP, the variants are produced by the “choice” of one exon among several possibilities for two internal regions of the protein. This process, novel in *MRPs*, could enable 14 different forms of the *Drosophila* protein.

[069] Exon duplication is a mechanism for the evolution of protein function, and in the case of the dMRP the duplication and variation of exons 4 and 8 surely provide *MRP* proteins with new properties. These alterations currently provide a series of isoforms with limited, defined changes in the large protein scaffold. Understanding the substrates and physiology of the different isoforms should provide new information on the role of these variable regions in *MRP* function in the fly, but also in human (and other organisms) for which the corresponding regions should retain attention in future structure/function studies.

[070] The capacity to produce a range of transporters with different specificities could be important for survival of the fly, by reducing the toxicity of an enlarged number of natural products encountered during its life. A function as important as protection from environmental toxins should not be based on a single molecule, but rather on a network of interacting enzymes with different reactivities. *MRPs* have a preference for

glutathione conjugated substrates, and in insects glutathione S (GS)-transferases have been shown to be involved in insecticide metabolism (Tang & Tu, 1994; Ranson et al., 1997; Hemingway & Ranson, 2000). Thus, MRPs could act together with the GS-transferases to confer insecticide resistance, by coupling toxin/conjugate efflux (MRP) to toxin conjugation (GST). This hypothesis is in line with the synergistic effect of an overexpression of both a GS-transferase and MRP1, leading to high-level resistance to the cytotoxic action of several drugs in a human cell line (Morrow et al., 1998a; 1998b).

ANOPHELES GAMBIAE

Materials and methods

Screening of the cDNA library and sequencing of the cDNAs

[071] The *cDNA* library from *Anopheles gambiae* G3 fourth instar larvae in λ Zap express vector (Stratagen) was kindly provided by Dr Hans-Michael Müller. 300,000 independent clones were transferred to Hybond N⁺ membranes (Amersham) and screened for *gMRP1a-d* by use of two PCR derived probes specific to *gMRP1a* (primer set *gMRP1for/gMRP1rev*, 645 bp) and *gMRP1b-d* (primer set *MRP104/MRP105*, 773 bp). Nucleotide sequences of the primers are shown in Table 4.

TABLE 4

Sequences of primers used in study			
SEQ ID NO:	Primer name	Primer sequence	Sequence specificity
43	gMRP1for	GGAGCGATTGTGATTTAC	<i>gMRP1a</i>
44	gMRP1rev	CTGGCCAACGGAAAGGTT	<i>gMRP1a</i>
45	MRP104	GGTCGAGTTCAAGGACTATC	<i>gMRP1b-d</i>
46	MRP105	TGAGTCACACTCCGCTACCA	<i>gMRP1b-d</i>
47	gMRP2for	GGAGCTCTGGTTGTGTTT	<i>gMRP1b</i>
48	gMRP3for	GGAAGTTGTGTCATCTTG	<i>gMRP1c</i>
49	gMRP4for	GGCAGCTTGGTGATCCTG	<i>gMRP1d</i>
50	gMRP234rev	TGACCGACCGAGAGGTT	<i>gMRP1b-d</i>
51	Actinf	CACACAGCATTTTTACAGCT	<i>Actin</i>
52	Actinr	TCTTTCATTGCCGCCGTATT	<i>Actin</i>

[072] The probes were ³²P-labeled (ICN) by random priming, and the library was screened following the Stratagen Zap express vector kit instructions. Forty eight positive plaques were purified by two rounds of screening and identified as containing *gMRP1a, b, c or d* by PCR with primers specific to each sequence (gMRP1for/gMRP1rev, gMRP2for/gMRP234rev, gMRP3for/gMRP234rev, and gMRP4for/gMRP234rev, respectively; Table 4). The size of the cDNA inserts was analysed by PCR with T7 and T3 universal primers. The cDNA inserts from seven positive clones that were longer than 5kbp were sub-cloned into the pBK-CMV vector by *in vivo* excision from the recombinant λ Zap express vector following the manufacturer's instructions. Four cDNA inserts representing each of the *gMRPs* were then sequenced by Genome Express (Paris, France).

Sequence analysis

[073] The *ClustalW* (Higgins & Sharp, 1988) program was used for sequence alignments. Tmap and Predictprotein were used for transmembrane domain search and Needle for pairwise sequence comparisons. Tmap and Needle are part of the EMBOSS software package from EMBL. The BLAST searches were done using the Washington University version of BLAST (Altschul et al., 1997). The softwares, except for the Predictprotein server (<http://dodo.bioc.columbia.edu/pp>), were accessed through the Institut Pasteur web site (<http://bioweb.pasteur.fr>).

Extraction of total RNA and cDNA synthesis

[074] Total RNA was isolated from adult mosquitoes using Tri Reagent (Sigma) according to the manufacturer's instructions. Total RNA from tissues (salivary glands, Malpighi tubules, digestive tract from midgut to hindgut, heads and thorax) was isolated using a protocol conceived for small tissue amounts : tissues were homogenized in liquid nitrogen before dissolution in 200 µl of RNABle (Eurobio). Twenty µl of chloroform were added and samples were incubated on ice for 15 min after vortexing. They were centrifuged 45 min at 15000 g and 4°C. Eighty µl of supernatant were recovered and mixed to two volumes of 100% EtOH. After 2 min of incubation at RT, they were centrifuged 5 min at 15000 g. Pellet was washed in 180 µl of 70% EtOH, and finally recovered in 22 µl of DEPC H₂O. The cDNA was synthesized in a 20 µl reaction mix containing 10 µg of total RNA, 1X AMV reverse transcriptase buffer (Promega), 10 U of AMV reverse transcriptase (Promega), 40 U of RNasin (Promega), 4 µg of random

hexanucleotide primers (Genset), and 1 μ M of each dNTP (Pharmacia). The reaction was incubated at 37°C for 1 H, and stopped at 95°C for 5 min.

Amplification on cDNA

[075] Primers used for specific amplification of *gMRP1a-d* on total cDNA are shown in Table 4. The cycling conditions were 94°C (2 min) for 1 cycle; 94°C (1 min), 56°C (1 min 30 sec) and 72°C (2 min) for 35 cycles (except with the actin primers, for which only 25 cycles were processed); 72°C (10 min) for 1 cycle.

Identification of A. gambiae MRPs

[076] Before *the* consortium was decided on the systematic study of the *A. gambiae* genome, sequences already available at the Genoscope (Evry, France) were used in a comparison to the human MRP1 protein sequence, and the entirely sequenced BAC clone 22C14, containing four successive sequences with similarity to that of the human were so identified. This BAC had been previously mapped to chromosome arm 3R, on position 30D.

Sequencing of the cDNAs and analysis of intron-exon boundaries

[077] In view of identifying cDNAs specific to each of the four *gMRP* genes, a cDNA library of *Anopheles gambiae* was screened as described above. The cDNAs selected from the library were systematically sequenced for comparison with the genomic sequence available from BAC 22C14. The resulting gene structure for each *gMRP* is shown schematically in Fig. 5. Length of exons and introns, their location on the gene and intron-exon junctions are shown in Table 5.

[078] The sizes proposed for the first exons are only putative, as they correspond to the longest cDNA found in the library, which appears consistent with a clustal W alignment of deduced amino acid sequences of several *MRPs*, and presents a favorable environment for the putative first methionine (Kozak, 1996).

Table 5. Organization of exon-intron junctions in the *gMRPs*

Exon							Intron		
Name	Location on protein sequence ^a	Size (bp)	3' acceptor ^b	5' donor ^b	Name	Phase	Size (bp)		
<i>MRP1a</i>	1	165		CCCTTG/gtgaga	1	0	83		
	2	10	gtacag/GTGGAC	TCCTTG/gtaagc	2	0	202		
	3	88	cttcag/GTGGGC	GCTGAG/gtaagt	3	2	224		
	4	277	atatag/ATTACT						
<i>MRP1b</i>	1	ND ^c		TTTTTG/gtaagt	1	0	603		
	2	13	ttacag/GACGAT	GCTTAT/gtaagt	2	0	76		
	3	113	ttcag/ATTATG	ATACCA/gtaagt	3	2	63		
	4	804	ctctag/GGAACT	CTTCAG/gtatgt	4	2	73		
	5	1315	ttccag/AATTGT	ATTCAG/gtaaga	5	0	65		
	6	1441	acacag/AAAACA						
<i>MRP1c</i>	1	418		GCTTAT/gtgagt	1	0	69		
	2	113	atttag/ATCGAC	GATGCA/gtaagt	2	2	96		
	3	334	ttatag/AGAACT	TTATCA/gtaagt	3	2	61		
	4	833	ttttag/GGAACT	ATGAAG/gtaagt	4	1	60		
	5	858	tttcag/AAATAT	CTTCAG/gttagt	5	2	63		
	6	1315	atctag/AATTGT	ATTCAG/gtgaga	6	0	71		
	7	1442	ttacag/AAAACA						
<i>MRP1d</i>	1	ND ^c		GCTTAT/gtgagt	1	0	69		
	2	113	atttag/ATCGAC	CATGCA/gtacgt	2	2	110		
	3	334	tcgcag/AGAAAT	ATACCA/gtagt	3	2	65		
	4	833	tttcag/ACAACT	AAGACG/gtaggt	4	1	98		
	5	859	caccag/AAATTA	CTTCAG/gtatct	5	2	73		
	6	1317	ttccag/AATTGT	ATTCAG/gtaaga	6	0	65		
	7	1444	ccacag/AAAACA						

a) The numbering is based on amino acid one being the putative first Met.

b) Capital letters are used for the sequence in the exon and small case letters for sequence in the intron.

c) Not Determined.

[079] For *gMRP1b* and *gMRP1d*, sequences could be amplified by RT-PCR upstream of the longest cDNA, and no size was proposed for the first exon in this case. The longest exon is exon 4 for *gMRP1a* (3638 bp), exon 2 for *gMRP1b* (2144 bp), exon 3 for *gMRP1c* (1497 bp), and exon 3 as well for *gMRP1d* (1497 bp). The smallest seems to be exon 1 (165 bp) for *gMRP1a*, exon 4 for *gMRP1b* (382 bp), exon 4 for *gMRP1c* (77 bp), and exon 4 for *gMRP1d* (80 bp). The mean size of exons is 1151 bp, 1119 bp, 671 pb, and 726 bp for *gMRP1a*, *gMRP1b*, *gMRP1c*, and *gMRP1d*, respectively. Introns are small in all four *gMRPs*, with mean sizes of 170 bp, 69 bp, 70 bp, and 80 bp for *gMRP1a*, *gMRP1b*, *gMRP1c*, and *gMRP1d*, respectively. These data contrast with those of the human MRP1 (Grant et al., 1997), for which exons are small (not greater than 311 bp), while introns are very large (from one to several kbp). Even when compared to the *Drosophila* structure, the *gMRPs* contrast by the fact that their exons are longer than their introns. Nevertheless, intron-exon boundaries share the characteristics of other eukaryotes. The acceptor sequence is characterized by a (t/c)ag motif, and the splice donor sequence is gt(g/a)ag(t/a), with a gt in 100%, (g/a) in 95%, and ag(t/a) in 74% of the cases. Introns 1 and 2 of *gMRP1a* are of class 0, while intron 3 is of class 2. Among the four introns in *gMRP1b*, two are of class 0, and 2 of class 2. Three of the six introns of *gMRP1c* are of class 2, two are of class 0, and one of class 1. In *gMRP1d*, the pattern is identical to that of *gMRP1c*. Interestingly, the class 2 introns are predominant in these genes, while *MRP1* and *dMRP* have more class 0 introns. A common feature, however, is the few number of

class 1 introns (only one in *gMRP1c* and *gMRP1d*, and none in *gMRP1a* and *gMRP1b*). Analysis on aligned sequences shows conservation of splice site locations and class of introns between the four *gMRPs* (Fig. 5), some data consistent with a common origin of these copies by duplication of an ancestor gene. Only the first splice site in *gMRP1a* has no equivalent in the three other *gMRPs*. *gMRP1c* and *gMRP1d* share exactly the same splice site, thus reinforcing the similarity between these two forms, which are also the closest at the level of their nucleotide sequence (Table 6).

[080] (a) Capital letters are used for the sequence in the exon and small case letters for sequence in the intron.

[081] (b) The numbering is based on nucleotide one being the A of the initiation codon. *gMRP1b* lacks two sites in comparison to them, and *gMRP1a* lacks three sites. But each of them also has one site at the 5' end of the sequence which is not present in *gMRP1c* and *gMRP1d*. Among all these sites, some are shared by either *MRP1* or *dMRP*, but interestingly there is only one site (the first site in *gMRP1b*, which is not shared by other *gMRPs*), which is also common to *dMRP* and *MRP1*, while these two genes also have some common splice sites. It is noteworthy that conservation inside the anopheline species and between this one and the others involves in nearly all cases splice sites that are present in *gMRP1c* and *gMRP1d*. This indication is clearly in favor of these two forms being the most related to the common ancestor of *gMRPs*, with *gMRP1a* and *gMRP1b* having lost some splice sites after their divergence. Identity/similarity comparisons between *MRP1* and *gMRP* deduced amino acid

sequences also show that *gMRP1a* and *gMRP1b* are the most divergent (Table 6). *gMRP1a* is the gene that has the most different sequence and has lost the most splice sites in comparison to the others, an observation that suggests that it has diverged the first from the primitive sequence. The second gene to be duplicated must have been *gMRP1b*, but based on this data, it is not clear if it is *gMRP1c* or *gMRP1d* that gave birth to the others. These two genes have exactly the same splice sites, and their identity/similarity values with other MRPs are not significantly different, so that it cannot be determined which of these two sequences is closest to those of other species. Even if *gMRP1a* was the first duplicated copy in the anopheline, it is difficult to explain its high degree of divergence, that leads its deduced amino acid sequence to share no more identity with other gMRPs than with those of other species. One explanation could be that this duplicated copy was able to by-pass the selection pressure controlling the ancestral gene evolution by acquiring slightly new functions, thus allowing evolution on a new way.

TABLE 6. Identity and similarity between gMRPs and some other MRPs

	Percent identity/similarity							
	gMRP1b	gMRP1c	gMRP1d	dMRP	MRP1	MRP2	MRP3	CeMRP1
gMRP1a	30.1/48.4	29.8/48.8	30.3/49.2	29.7/47.0	29.3/46.1	29.2/47.6	30.1/46.0	29.3/45.6
gMRP1b		78.3/88.9	79.7/89.4	52.9/71.2	48.4/66.8	45.2/65.2	48.2/65.4	45.2/63.1
gMRP1c			88.2/94.4	53.7/70.9	48.7/66.8	45.0/64.7	47.1/65.4	44.3/62.2
gMRP1d				53.0/71.2	48.7/66.9	44.5/64.6	45.9/65.0	44.5/63.1
dMRP					59.9/68.2	46.8/65.8	50.6/67.2	46.4/63.8
MRP1						48.7/65.9	56.9/74.0	46.5/63.0
MRP2							46.7/65.3	42.2/60.4
MRP3								45.5/62.6

Percent identity and similarity between pairs of sequences was calculated using the Needle program, with gap opening and extension values of 5 and 1, respectively. The amino acid sequences are as follows : gMRP1a, *A. gambiae*; gMRP1b, *A. gambiae*; gMRP1c, *A. gambiae*; gMRP1d, *A. gambiae*; dMRP, *D.melanogaster*, MRP1, human; MRP2, human; MRP3, human; CeMRP1, *C.elegans*.

Characterization of the deduced amino acid sequences

[082] The *predicted* amino acid sequences of the four *A. gambiae* MRPs deduced from the sequencing studies are shown in Fig. 6, aligned with the *Drosophila* dMRP and human MRP1. For any of the *A. gambiae* are we sure of having entire cDNAs, so we do not know with certainty which is the initiation codon; however, based on comparison of the deduced amino acid sequences, we predict that the first methionine, for each gMRP, could be the one indicated in Fig. 6. In each case, the chosen methionine is preceded by STOP codons in all three ORFs, without other identical residue (data not shown). *gMRP1c* and *gMRP1d* also share an A nucleotide in position – 3, in accordance with Kozak's observations on translation start sites (1996).

[083] Alignment of these anopheline MRPs with those of other animal organisms clearly showed a difference between data obtained for gMRP1a and those of the three other gMRPs. gMRP1b, c, and d share more homogeneous sequences, while gMRP1a is strikingly different. Its identity and similarity values with other MRPs are low, even with the three other gMRPs (Table 6). The mean identity and similarity values between gMRP1a and the three other gMRPs are 30.1% and 48.8%, respectively, and they are of 29.5% and 46.5% with other MRPs. In contrast, gMRP1b – d are much more related to one another (82.1% mean identity, and 90.9% mean similarity) than to other MRPs (48.4% mean identity and 66.2% mean similarity). Among these, dMRP is the closest (53.2% mean identity and 71.1% similarity), while among the human MRPs, it is MRP1 (51.4% mean identity and 67.2% mean similarity). Identity between MRP1 and gMRP1b-d is even greater than between MRP1 and MRP4 or MRP5 in the human

species (Borst et al., 2000). MRP3, the sequence of which is close to that of MRP1 (Borst et al., 1999) also shares great identity/similarity with the gMRP1b – d (47.9%/65.7% respective mean values). gMRP1c and gMRP1d are those which match the best with other sequences analysed here. It is then probable that one of these two sequences gave birth to the three others by gene duplication. Despite the great divergence of gMRP1a, the sequences of the gMRPs are more conserved between themselves than are those of the human MRPs integrated in this study (56.1% mean identity, and 69.8% mean similarity for the first ones versus 50.8% mean identity and 68.4% mean similarity for the second). The best matches between gMRPs and dMRP agrees with the logical hypothesis of a common ancestor gene for the two insects.

[084] Fig. 6 highlights that the more conserved regions are the NBDs, the MSDs sharing less similarity. The less conserved regions are the first MSD and the linker region, as usual in ABC transporters (Hipfner et al., 1999). Walker A and B motifs, as well as the ABC signature are clearly identified on Fig. 6. gMRPs share the distinctive features of MRP1-like proteins, that are a structure with three MSDs in place of two for most of the ABC transporters, and the lack of 13 amino acids in the first NBD, in comparison to P-glycoprotein (Hipfner et al., 1999), which confirms their relatedness to MRP1 rather than to other structurally close proteins.

[085] The deduced amino acid sequence of *gMRP1c* was compared to the sequences contained into the Sptnrndb database using the BLASTP program, and the result showed best scores with SD07655 and CG6214 (1527 and 1507, respectively),

which are sequences from the *D. melanogaster* MRP1 homologue (Grailles et al., submitted paper). The next best scores are those of the murine MRP (1338) and human MRP1 (1335). On the other hand, when the sequence was compared to the anopheline databases from TREMBL and SWISSPROT (<http://konops.imbb.forth.gr/AnoDB>), the highest scores observed were much lower than those cited above; they were not greater than 108 for SWISSPROT, and 67 for TREMBL. These results, in accordance with *in situ* hybridization, suggest that no other paralogues than those described here exist in the anopheline genome.

[086] Comparison between the four cDNAs and the genomic sequence represented by BAC 22C14 allowed us to evaluate the length of each gene to slightly more than 5 kbp. *gMRP1a-d* respectively contain 2, 5, 7, and 7 exons. Contrary to the *Drosophila* gene *dMRP*, these ones do not possess exons represented as multiple interchangeable copies, and the introns are small (the biggest is only 603 bp), and these reasons explain the great difference observed between these genes length and that of the *Drosophila* or human homologues (22 kbp and 200 kbp, respectively).

Comparison of the four copy structure and expression

[087] In human, the predicted structures of MRP proteins distinguishes two sub-groups : the first one contains MRP1, 2, 3, 6 and 7, which possess three MSDs, while the *second* group, containing MRP4 and MRP5, has a classical ABC-transporter structure with 2 MSDs (Hopper et al., 2001). The topological predictions we have realized for our gMRPs are in favor of three MSDs for these proteins, which means that they may belong to the first group. This is in accordance with their sequence similarity

with the genes of this group, which is more important than with those of the second group (data not shown). The combination of secondary structure predictions and biochemical analyses have led to a topological model of MRP1 with 17 transmembrane domains, five in MSD1, and six in MSDs 2 and 3 (Kast & Gros, 1998). Also, the two NBDs have been clearly localized to the intracellular compartment by several methods (Flens et al., 1994; Bakos et al., 1996; Hipfner et al., 1996, 1997; Kast & Gros, 1998). Although we have no biochemical data on the gMRPs that could help in predicting their membrane topology, we assume, in regard to their relatedness with MRP1, that they should have a similar organization. This means that the NH₂ extremity of these proteins is more probably external, and the two NBDs are internal, as well as the COOH extremity. Respecting these features imposes some constraints on the organization of the transmembrane helices. It means that MSD1 must have an odd number of transmembrane domains, while MSD 2 and 3 each have an even number of these. We tried to integrate these data on the analysis of the secondary structure predictions obtained for our sequences, using the Tmap and PredictProtein softwares. Taking into account the results of these programs and known features on MRP1 structure, the first MSD of our gMRPs could contain five transmembrane helices, while MSD2 and MSD3 could each span the membrane six times. Such predictions are in accordance with the structure predicted for MRP1.

TABLE 7. Expression in Different Tissues

Gene	Date	Tissue			
		Salivary gland	Malpighian Tubes	Digestive Tube	Thorax
gMRP1a	18/10/01				+++
	22/10/01				+++
	25/10/01	0	+++	+++	+++
	1/03/02	+++	++	+++	
	8/03/02	++	+++	+++	++
	22/03/02	+++	++	+++	+++
gMRP1b	18/10/01				+++
	22/10/01				+++
	25/10/01	0	++	+++	+++
	1/03/02	+++	0	+++	
	8/03/02	+++	+	+++	++
	22/03/02	+++	0	+++	+++
gMRP1c	18/10/01				+++
	22/10/01				+++
	25/10/01	0	+++	+++	+++
	21/03/01	+++	+	++	++
	21/03/01	+++	+++	+++	
	22/03/02	+++	+	+++	+++
gMRP1d	18/10/01				+++
	22/10/01				+++
	25/10/01	0	++	+++	+++
	1/03/02	+++	+	+++	
	8/03/02	+	+++	+++	++
	22/03/02	+++	0	+++	+++

[088] In an attempt to see if each of these four genes could be related to a specific function, we compared their relative expressions between different tissues. This was *performed* by semi-quantitative RT-PCR, as described above. The tissues used were thorax, heads, Malpighi tubules, digestive tract, and salivary glands. After extraction of the RNAs from these tissues, reverse transcription (RT) was performed, followed by PCR with primers specific to genes *gMRP1a-d*. Table 7 shows that cDNAs corresponding to all four genes were present in these tissues. At least four experiments were conducted on each tissue. Comparison of the relative expression of the four *gMRP* genes in each tissue did not show differences of expression inside the salivary glands, the digestive tracts, and the thorax. However, in the Malpighi tubules we could note a stronger expression of *gMRP1a*, and a lesser expression of *gMRP1b*. At the head level, *gMRP1b* was always strongly expressed, while *gMRP1c* and *gMRP1d* exhibited a weak expression. Our results are in favor of an ubiquitous localization of the four gMRPs, but the differences of expression observed could be related to functional specificities acquired by gene duplication evolution.

[089] In summary, *Drosophila melanogaster* has a gene very similar to human *MRP1* that encodes a full ABC-transporter containing three membrane-spanning domains (MSDs) and two nucleotide-binding domains (NBDs). This 19 exon insect gene, *dMRP* (FBgn0032456), spans slightly more than 22 kb. The cDNA SD07655 representing this gene was sequenced and found to contain sequences from 12 exons including single copies of two exons having multiple genomic copies. The gene

contains two variant copies of exon 4 and seven of exon 8. While a cDNA contains only one version of each variable exon, all forms of these variable exons were detected in adult fly mRNA. These results predict that *Drosophila* could make 14 different MRP isoforms from a single gene by substituting different variable exons. This is the first report of any organism using differential splicing of alternative, internal exons, to produce such a large array of MRP isoforms having the same size, but with limited and defined internal variations. Defining the functional differences in the dMRP isoforms should provide clues to the structure/function relationships of the amino acids in these MRP domains, both for the insect enzyme and for those of other species.

[090] The invention also constitutes the description of *MRP* homologues found in the *Anopheles gambiae* mosquito by computational analysis of genomic sequences. cDNAs corresponding to these genes were produced, and compared to the genomic sequences, in view of determining their structure. The particular interest of this study is the existence, in the anopheline genome, of a cluster of four genes of the same family, two of them having very close sequences. It does not seem that other copies of this gene family exist elsewhere in the genome, and the four described here, which have probably emerged by gene duplication, could be characterized by specific physiological roles, due to some differences observed in their tissue expression.

[091] It is now clear, in regard of the increasing knowledge on human MRPs, and also on other animals, that this family of proteins is involved in a variety of biological processes, explaining the diversity of forms encountered in the organisms. In the only

other insect studied for MRPs, *Drosophila melanogaster*, it seems that there is a unique gene, with internal variations at the exon level. In the case of the anopheline, no such variable exons have been detected, but genetic diversity was introduced by duplication. Particularly striking is the divergence observed in the *gMRP1a* paralogue in comparison to the three other genes. Even if it is not clear how to interpret this fact, it can be assumed that acquisition of a new function important for the mosquito could have explained a high rate of sequence divergence from the other paralogues during evolution.

[092] RT-PCR experiments have demonstrated the transcription of each of the four forms in the adult mosquito, in the different tissues examined. Differences were noted in the level of expression of some forms on certain tissues, and also on a mosquito cell line, where it was clearly observed that *gMRP1a* and *gMRP1b* were far less expressed than the two others. These observations agree with the hypothesis that the different forms should have different physiological roles.

[093] Logically, one can expect that the physiological function of these proteins will be similar to that of the human MRP1, because their structures are close. Even closer is the *D. melanogaster* dMRP, and data on that protein function will probably greatly help in the study of the anopheline forms, when they are available. It has been recently shown that dMRP could transport the well known substrate MRP1, leukotriene C4 (Roger Deeley, personal communication), which is the first functional demonstration of the relationship between the drosophila and human proteins. In *D. melanogaster*, the

existence of variable exons has led us to suspect that these regions could play an important role in the structure and/or function of the protein (results under submission). The comparison of the sequences in this region, between the fly and human MRPs, showed that some amino acids were highly variable in some otherwise conserved regions. When we compare the alignment between gMRPs, MRP1 and dMRP in the same regions, this variability is confirmed. It seems to us that functional studies on these gene products should particularly focus on these amino acids, because they could lead to different substrate affinities. As the four forms of gMRPs were not equally expressed in all tissues one can speculate that their different specificities could involve them in different biological functions. Biochemical studies should help answer these questions in the future. The region corresponding to exon 4 of *dMRP* has not yet been studied functionally, but in the region corresponding to exon 8 one amino acid of the human *MRP1* has been shown to be important in transport of anthracyclines, vincristine, and VP16 (Zhang et al., 2001). This residue is highly polymorphic in the gMRPs, as well as another residue, corresponding to Thr¹²⁴² of MRP1, which is also important for the transport of several substrates in human. The variability at this level is probably related to functional specificity of the 4 gMRPs. Also, it is interesting to note that a nearby amino acid, Trp¹²⁴⁶ of MRP1, which functional importance has been shown (Ito et al., 2001), and which is very conserved in the ABCC group (the MRPs group inside ABC transporters), is not conserved in any of the four gMRPs. Instead, gMRP1a has a Ser

residue at this position, and the three others a Phe. This observation must be of physiological importance.

[094] MRPs are transporters of organic anions, either conjugated or not to acidic ligands such as reduced glutathione (GSH), glucuronate or sulfate. Very little is currently known about the physiological function of MRPs, but the different members of the family in human have been characterized for different substrate specificities, which are most probably related to different physiological functions (Borst et al., 2000). Likewise, two homologue genes can have different substrate specificities from one species to another, which is illustrated for example by the human MRP1 and murine *mrp* (Stride et al., 1999). It is thus very probable that the variations observed in otherwise very conserved regions in the anopheline MRPs are related to such specificity. It is striking that, although three of the *gMRPs* have very similar sequences, and gene organization (especially *gMRP1c* and *gMRP1d*), the fourth, *gMRP1a*, is very different, in structure as well as in organization. It is too early to speculate about the implications of this observation, but is worth noting that this form exhibited different levels of expression according to the tissue or cultured cell line analysed. Probably, the different forms of anopheline MRPs act as members of a network of detoxifying proteins which clear the cell from the variety of products encountered in the environment.

[095] One particular aspect of these protein function that has kept our attention is their strong *affinity* for glutathione conjugates, because it has been shown that glutathione *S* (GS)-transferases were involved in insecticide metabolism in the

anopheline mosquito (Tang & Tu, 1994; Ranson et al., 1997; Hemingway & Ranson, 2000). It is so possible that the two proteins act together in a biological process that would couple conjugation of the toxic products to exportation of the newly formed complex. This hypothesis is supported by results on human cell lines that have shown a synergistic effect between GS-transferase and MRP1 overexpression to confer high-level resistance to several drugs (Morrow et al., 1998a; 1998b).

[096] Gene duplication is one of the mechanisms contributing to evolution by conferring new *properties* to a protein, or even new function. This is more probably the case with the four gMRPs described here. Changes that have appeared in the gene sequence after the different duplications seemingly have provided functional specificity to each of the isoforms, which is relevant with the differences observed in expression at the tissue level. It now remains to be determined which are the substrates of each isoform, and how these contribute to the physiology of the mosquito; particularly interesting will be to investigate their potential role in insecticide resistance, knowing that insecticide control of vector is currently the most successful strategy against malaria (Collins et al., 2000). A better understanding of the mechanisms involved in insecticide resistance will help developing new control measures in the future.

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